Final Technical Report

Assessment of Regenerative Capacity in the Dolphin

Jeffrey M. Catania and Robert J. Harman

12860 Danielson Court, Suite B

Poway, CA 92064

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Catania, Jeffrey, M
Harman, Robert, J

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Vet-Stem, Inc.
12860 Danielson Ct.
Suite B
Poway, CA 92064
USA

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Office of Naval Research
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14. ABSTRACT
Described herein is the final technical information pertaining to a multi-year effort to determine and characterize the use of adipose (fat)-derived stem cells in the treatment of epidermal (skin) wounds. Adipose tissue was successfully harvested from the nuchal fat pad of six Atlantic Bottlenose dolphins via liposuction; cells released during the digestion of the adipose tissue were analyzed for cytology, assayed for the total number of colony-forming cells, expanded in culture, differentiated into multiple cell lineages, analyzed for stem cell surface markers and stem cell related genes. Cultured cells were also cryogenically frozen for autologous and allogeneic cell therapy treatment of dolphin skin wounds. Stem cells were incubated with white blood cells from non-donor dolphins and were shown to be non-immunogenic. Injection of stem cells along the wound axis into skin wounds of dolphins display a more rapid healing than carrier solution alone.

15. SUBJECT TERMS
Adipose Derived Stem Cells, Regenerative Cells, Marine Mammals, Atlantic Bottlenose Dolphin, Autologous Cell Therapy, Allogeneic Cell Therapy

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a. Scientific and Technical Objectives

There is no information available on the distribution and functionality of adult stem cells in adipose tissue in the dolphin. However, it has been reported that cutaneous wounds in the dolphin heal very rapidly. We hypothesize that adult stem cells play an important role in wound healing in the dolphin. The objective of this proposal is to assess various adipose tissue depots in the dolphin for the presence of nucleated cells, to characterize those cells in order to establish their regenerative capacity and to use isolated, regenerative cells for treating wounds in a wound healing model in the dolphin. Vet-Stem will use its extensive knowledge and technical expertise to optimize the isolation of regenerative cell populations from dolphin adipose tissue, to characterize those cells with cross reactive CD markers and primers, to differentiate the cells into specific lineages and assess the therapeutic benefit of applying concentrated doses of the regenerative cells to wounds in a wound healing model. The knowledge gained from these studies will support the potential development of off-the-shelf cell-based "products" for treating a variety of pathologies and disease states in dolphins in the Navy and could extend knowledge to the treatment of sailors and other military personnel.

b. Approach

We propose to use techniques and protocols developed at Vet-Stem to characterize the nucleated cell preparations obtained from dolphin adipose tissue. The characterization will confirm the existence of adipose-derived stem cells (ASCs) in the dolphin, by demonstrating that a subset of cells from the isolated cell preparations are plastic-adherent in cell culture and these cells can be differentiated with specific media into adipogenic, chondrogenic, osteogenic and neurogenic cell lineages. This will provide the initial evidence of ASCs in dolphins. Further phenotypic characterization will be completed by assaying the cells for key surface proteins with existing immunological and molecular biological reagents for ASCs from other species. Positive identification of these existing reagents using cultured dolphin cells will prove that dolphins contain ASCs in their tissues. Cryopreserved cultured ASCs will be used as autologous cellular therapy for dolphin skin wounds. Finally, the cells will be tested for immunogenicity to develop an allogeneic (same species, universal donor cell line) model of cell therapy in the dolphin. After establishing these safe methods, we will use autologous and allogeneic ASCs to assess the therapeutic impact of applying a concentrated "dose" of regenerative cells in the wound healing model.

c. Accomplishments

Identification of Suitable Adipose Depot in the Atlantic Bottlenose Dolphin

The first goal of this study was to determine a suitable adipose depot from which to harvest adipose. To compare outcomes from various adipose depots, adipose samples were collected from three separate locations during a dolphin necropsy; mandibular adipose, subcutaneous adipose, and blubber. Tissue samples were washed, trimmed, minced and then digested, as described further below, for a total time period of 50 minutes. Following digestion, the blubber contained a large amount of ligamenture which could not be digested, whereas the mandibular adipose and the subcutaneous adipose were readily digested. In this single set of comparisons, blubber contained the least number of cells per gram and subcutaneous adipose contained the highest number of cells per gram (Table 1). The total viable cells per gram for these tissues

were very low (<50,000 cells per gram) and is most likely due to the time of harvest from the dolphin necropsy. The dolphin echolocating apparatus consists of a unique composition of adipose, both on the outer edge of the dolphin lower mandible (jawbone) and in an area that is analogous to the cheek. The blubber is a specialized organ partially consisting of some adipose, which serves as an insulator to the cold water temperature and may contain a population of stem cells that will translocate based upon a sustained injury. However, adherent cells were isolated from the tissues, indicating these areas may be useful for future samples. More importantly, this data supports the use of subcutaneous adipose for isolating adipose-derived stem cells, an area in which Vet-Stem has considerable expertise.

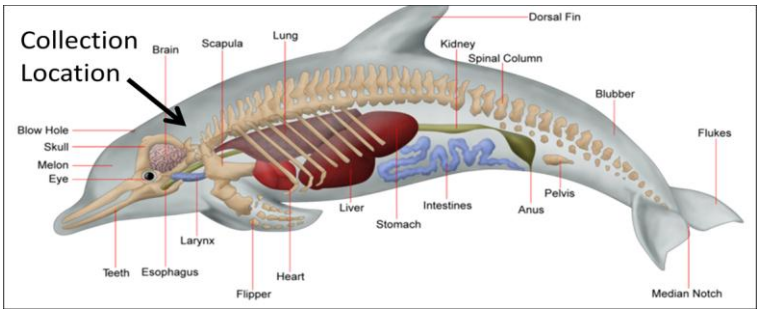
Two additional adipose-containing tissues have been identified and were compared against subcutaneous adipose, which served as an experimental control.

Table 1. Results from Multiple Adipose Depot Dolphin Necrospy Adipose Collections.

Accession Number	*Collection Location*	*Sample Weight (grams)*	*Total Viable Cell Yield*	*Viable Cells per Gram*	*Percent Viable Cells*
OBA-043010-01	Blubber	48.66	182,000	3,740	82.5
OBA-043010-02	Subcutaneous Adipose	31.92	1,484,800	46,516	87.1
OBA-043010-03	Mandibular Adipose	37.40	1,282,600	34,294	91.7

While there are multiple adipose depots within the Atlantic Bottlenose dolphin, following consultation with the NMMP veterinarians, the region identified as being the most likely to have a high density of subcutaneous adipose was the nuchal fat pad; an area analogous to the back of the human neck (Figure 1). The use of ultrasound was deemed the optimal method to verify the location, measure the density and aid in the placement of the liposuction cannula in the adipose depot. The difference between the various layers of muscle and adipose tissue is readily visible on the ultrasound and aids in the progression of the cannula to the adipose field (Figure 2). The actual liposuction technique is largely based on Vet-Stem's experience from equine (horse) liposuction collections.

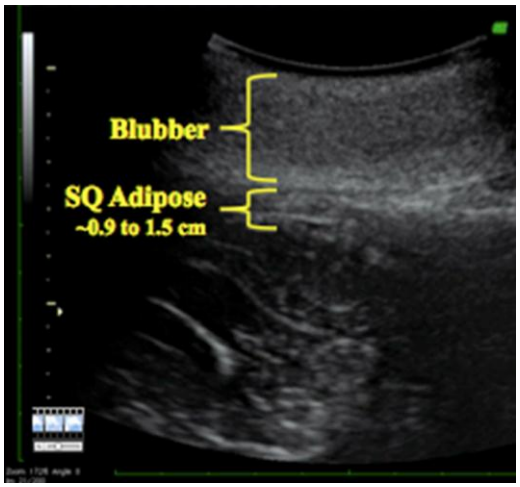
Figure 1. Anatomical Diagram of the Atlantic Bottlenose Dolphin.



Collection of Adipose from the Atlantic Bottlenose Dolphin

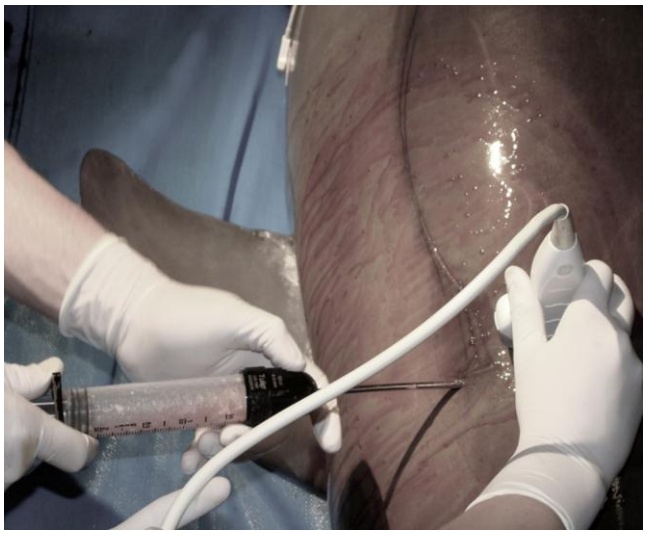
Following transport to the surgical suit, the dolphin is allowed to stabilize while respiration and heart-rate monitors are attached. The animal is given a local skin block (lidocaine) in the approximate area where the infusion cannula will be inserted. An infusion cannula with attached syringe is then used to infuse the intended adipose harvest site with tumescent solution; a solution consisting of lidocaine, epinephrine and saline. A waiting period of between 10 and 20 minutes is then required to ensure analgesia; during this time the collection area is massaged to diffuse the tumescent solution evenly throughout the area and assist in the disruption of the adipose depot connective tissue. After the designated waiting period, a harvesting cannula is attached to a 60 milliliter Toomey syringe and the cannula is then inserted into the same entry port used for the infusion. The liposuction is performed by applying a vacuum to the syringe, monitoring the progression of the cannula to the collection area (Figure 3) and continuously moving the cannula to assist in the harvest. Throughout the entire procedure, ultrasound imaging is used to ensure animal safety and proper identification of the subcutaneous adipose depot. The duration of the liposuction procedure is controlled by the NMMP veterinarians and is typically completed in less than 40 minutes from the time of infusion, the time at which the analgesia from the tumescent solution begins to wear off. The method described herein has resulted in the safe collection of adipose from the six dolphins; no adverse events related to this procedure have been observed or reported.

Figure 2. Ultrasound Identification of tissue layers in the dolphin.



The subcutaneous adipose depth was measured during each procedure and ranged from 0.7 to 1.5 cm. As per the dolphin adipose liposuction collection schedule outlined to us by the NMMP IACUC, a total of six dolphins had adipose tissue collected via liposuction, 4 males and 2 females. The first collection performed on 02 December 2009 and the final collection was performed on 16 March 2010. Adipose sample collection information is shown later in Table 1. An additional tissue collection was performed during a dolphin necropsy on 30 April 2010. A total of six NMMP personnel, four veterinarians and two veterinary technicians, have been trained on the procedures outlined in our Quality-approved training aid, Dolphin Tumescent Liposuction Aid. NMMP veterinarians trained, supervised and assisted one another, demonstrating that this procedure could readily be deployed with field-training.

Figure 3. Photograph of Ultrasound-guided Liposuction.



An area of improvement for the harvesting procedure includes the use of larger diameter infusion and aspiration cannula to efficiently traverse the cannula through the various layers of epidermis, blubber and adipose. A larger cannula would allow for more rigidity in the wall of the cannula, which could lead to more consistent harvests of adipose tissue from the dolphin. A key aspect to the collection procedure is the relationship of the angle of the harvesting cannula to the adipose depot; this angle should be as shallow as possible to keep the harvesting cannula within the adipose depot for optimal harvest (see Figure 3). A more rigid cannula would allow more control of this procedure.

Using ultrasound-assisted liposuction is recommended as the optimal method to collect adipose from dolphins as the procedure is minimally invasive, has demonstrated safety and results in successful adipose harvests.

Digestion of Adipose Collected from Atlantic Bottlenose Dolphin

To optimize the procedure, we set out to monitor the progression of adipose tissue digestion including the use of a hemacytometer for monitoring the size of the particles in the digestate. However, particle size remained too large to properly view using a hemacytometer due to ligamenture and fibrous tissues collected during the liposuction process. Instead, qualitative observations made throughout the digestion process were used to optimize tissue digestion by removing the digestion tubes from the digestion chamber, observing the progression of digestion and stopping the digestion when appropriate. As we have seen with other species, outcomes vary greatly between animals. Therefore, there is not one set of optimal digestion parameters, per se, but rather a window of digestion time which yields both cells of high viability (greater than 65%) and high number of viable cells per gram of adipose (greater than 150,000 viable cells per gram). The data for adipose digestion is tabulated in Table 2. The average number of viable cells per gram was 357,485 with an average of percent viability of 77.7%.

Table 2. Liposuction Sample Information.

Accession Number	*Collection Date*	*Sample Weight (grams)*	*Total Viable Cell Yield*	*Viable Cells per Gram*	*Percent Viable Cells*
OBA-120209-10*	12/2/2009	6.30	546,000	86,667	86.7
OBA-012610-08	1/26/2010	12.76	3,280,000	257,053	88.2
OBA-021710-06	2/17/2010	4.62	1,546,200	338,571	75.2
OBA-022310-07	2/23/2010	5.89	4,097,700	695,705	83.1
OBA-030910-10**	3/9/2010	0.91	182,700	200,110	50.0
OBA-031610-11	3/16/2010	5.79	3,281,800	566,805	83.0
	Average	*6.05*	*2,155,733*	*357,485*	*77.7*
	Standard Dev.	*3.84*	*1,622,274*	*230,981*	*14.3*

*Sample was digested using a small adipose biopsy protocol. All other samples processed according to our standard procedure.
**Cell count was at the limit of detection; actual viability may be higher. A recount was not performed since all cells were placed directly into culture to maximize the cellular yield.

To briefly describe the digestion process, the lipoaspirate is centrifuged, the adipose is isolated, washed (Figure 4) and digested using a proprietary blend of enzymes over a time course of 20 to 25 minutes in length (Figure 5). The samples are then repeatedly washed and centrifuged to obtain a purified cell pellet (Figure 6). The total amount of adipose harvested via liposuction

from each dolphin varied, but to ensure the maximum number of cells could be released, each individual sample was qualitatively-observed throughout the digestion process and digestion was halted when the majority of adipose particles were no longer visible.

Figure 4. Photographs of Harvested Lipoaspirate and Isolated Adipose Tissue.

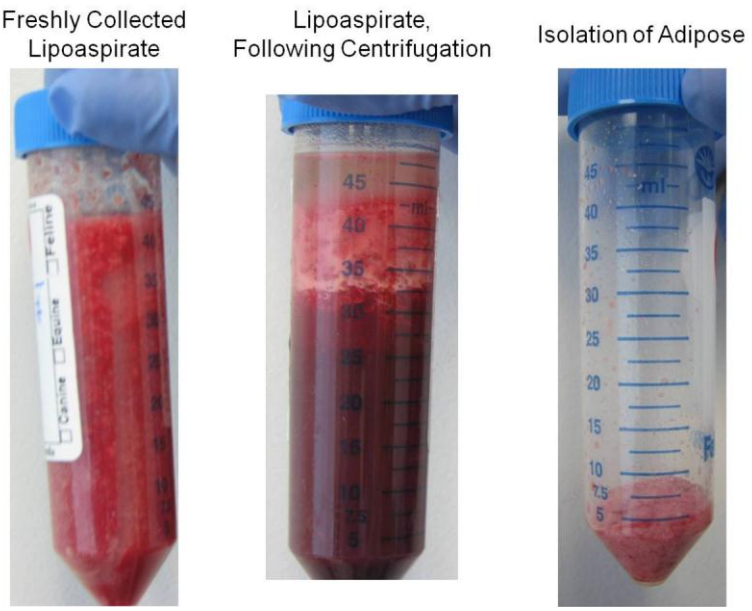
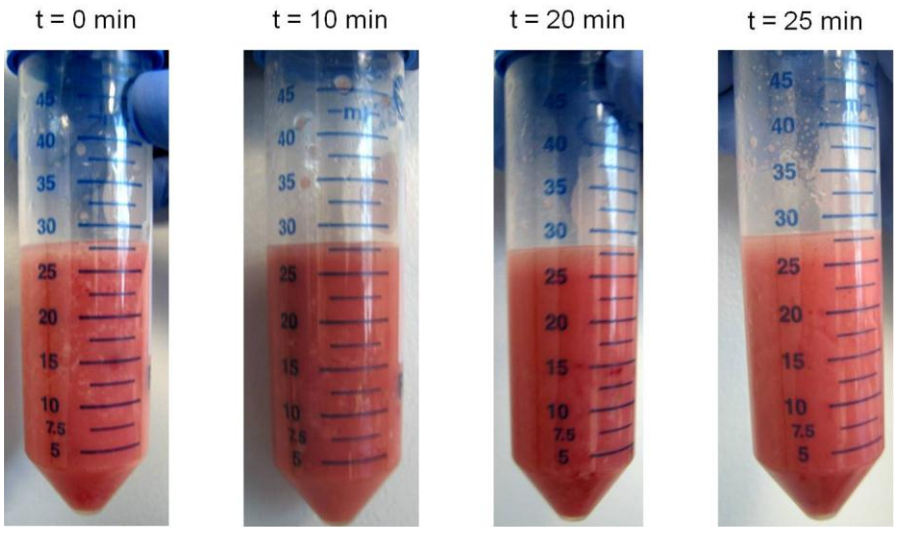
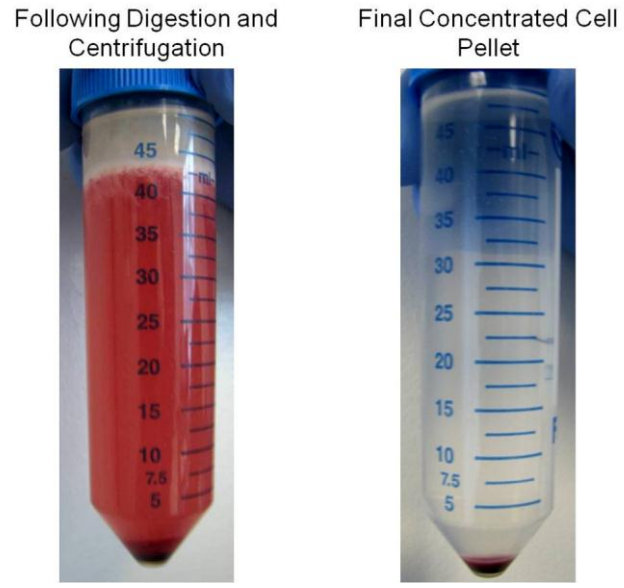


Figure 5. Photographs of the Digestion Process at Various Time Intervals.



The initial sample, OBA-120209-10, was processed differently from the remaining samples using a technique specifically developed for this contract. In brief, the adipose is centrifuged, isolated and washed, and then divided amongst multiple microcentrifuge tubes. Digestion cocktail (a proprietary blend of enzymes) was then directly added to each tube and the sample was warmed and agitated on a rocking platform. The total number of viable cells per gram for this procedure was lower than expected (86,667 viable cells per gram) and while the yield for this sample was sufficient for placing the cells directly in cell culture for expansion, all other samples were digested using our standard procedure with the previously described digestion process difference and resulted in an increased number of viable cells per gram (>200,000 viable cells per grams).

Figure 6. Completion of Adipose Digestion and Concentrated Cell Pellet.



With the exception of the initial sample, all dolphin liposuction collections have been processed according to Vet-Stem's liposuction protocol, with the addition of monitoring the digestion stop. This protocol has been used to successfully release cells from over 200 equine liposuction samples. Due to the large variability between animals, the recommended protocol for dolphin adipose tissue samples collected via liposuction is to use our commercial protocol up to the digestion process and then to directly monitor the adipose digestion to ensure completion of the digestion process, maximizing the number of cells released from the adipose.

Characterization of Cells Released from the Digestion of Dolphin Adipose

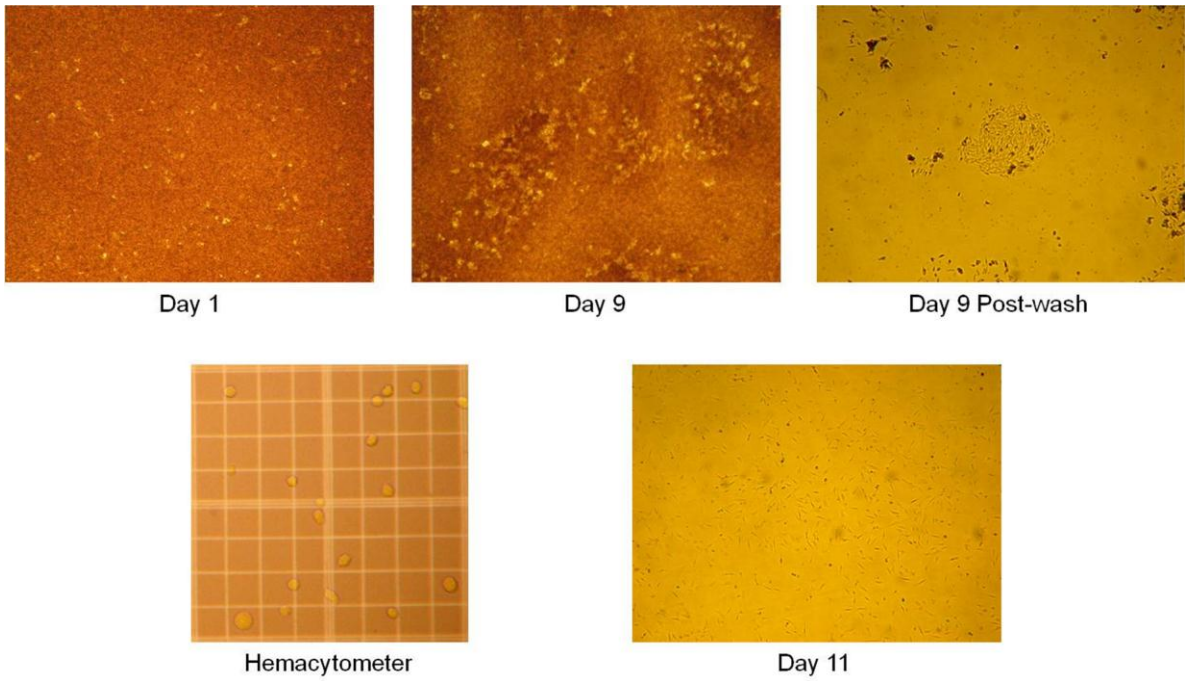
To characterize cells released from dolphin adipose during the digestion process, an hierarchical schema was employed to ensure that, at a minimum, dolphin cells could be expanded in culture to obtain enough cells to treat the dolphins autologously while characterizing the cells as best possible. Although not accepted universally, the International Society for Cellular Therapy (ISCT) has established minimal criteria for defining multipotent mesenchymal stem cells (Dominici M, et al., 2006):

1. Cells must adhere to plastic when placed in culture conditions;
2. Cells must express the Cluster of Differentiation (CD) markers CD73, CD90 and CD105 and lack expression of CD34, CD45, CD14, CD11b, CD79, CD19 and HLA-DR (major histocompatibility class II);
3. Cells must differentiate into adipocytes, chondroblasts and osteoblasts in vitro.

While these criteria are not universally accepted, they serve as the basis for the characterization of the cells isolated from the digestion of dolphin adipose. Throughout the term of this contract, Vet-Stem has employed and utilized the latest techniques and information to ensure scientifically relevant results, in addition to those recommended by the ISCT.

To begin demonstration that the cells isolated from the digestion of dolphin adipose are stem cells, nucleated cells were expanded in culture. Cells were seeded directly onto cell culture flasks at an initial seeding density of 13,333 cells per square centimeter. Since red blood cells are collected during centrifugation with the mesenchymal stem cells, the initial culture seeding contains a large number of red blood cells. Typical growth and passage observation of the stem cells is shown in Figure 7, which encompasses the typical culture at Day 1 through the first passage at approximately Day 10. The cultured cells measured from 11 to 29 microns, as calculated using a hemacytometer. Throughout the culture process, the cells remained the characteristic spindle-shape of cultured stem cells and remained as such throughout all 6 passages. Cells were expanded in mesenchymal stem cell media, subcultured when cells reached approximately 80-90% confluence and plated into new flasks at a minimum seeding density of 4,000 cells per square centimeter. The average doubling time for all dolphin cultures was found to be 1.14 +/- 0.31 days with individual culture doubling times ranging from 0.78 to 2.09 days (Table 3). All cultured cells which are to be used for autologous treatment have undergone rigorous sterility testing according to the United States Pharmacopeia <71> and Mycoplasma testing to ensure patient safety.

Figure 7. Cell Culture Expansion of Dolphin Stem Cells.



A “direct-to-culture” procedure was also performed, whereby a small volume of the undigested lipoaspirate was placed directly into culture media; cells were expected to slough off the adipose particles and grow directly on the culture flask. However, over the course of 2 weeks, no cells were observed to have adhered to the flask and the experiment was terminated.

When a cellular yield of sufficient quantity was obtained, cells released from the digestion of the harvested adipose tissue were submitted for cytological analysis. All cytology counts were performed on a Beckman Coulter AcT Diff hematology analyzer, which can discretely analyze the red blood cells and multiple white blood cell types. In all freshly-digested dolphin samples submitted for analysis, the percent of segmented neutrophils and lymphocytes is much higher

than would be expected (Table 4); it is suspected that the software analysis package has incorrectly categorized the cells and we are in the process of coordinating with the NMMP to obtain blood cell counts taken prior to or during the adipose harvest for comparison.

Table 3. Doubling Time of Dolphin Stem Cell Cultures.

Accession Number	Passage Number	Doubling Time (Days)	Average	Std. Dev.
OCP-120209-11	2	1.38	1.54	0.39
	3	2.09		
	4	1.16		
	5	1.79		
	6	1.27		
OCP-012610-09	2	0.98	0.96	0.18
	3	0.71		
	4	0.87		
	5	1.16		
	6	1.08		
OCP-021710-09	2	1.06	0.96	0.11
	3	0.78		
	4	1.04		
	5	0.95		
	6	0.93		
OCP-022310-09	2	0.87	1.03	0.17
	3	0.85		
	4	1.00		
	5	1.20		
	6	1.20		
OCP-030910-11	2	0.83	1.19	0.29
	3	1.14		
	4	1.05		
	5	1.33		
	6	1.61		
OCP-031610-12	2	1.27	1.18	0.28
	3	1.16		
	4	1.06		
	5	1.58		
	6	0.82		
Cultures Combined	***2***	***-***	***1.07***	***0.22***
	3	***-***	***1.12***	***0.51***
	4	***-***	***1.03***	***0.09***
	5	***-***	***1.34***	***0.30***
	6	***-***	***1.15***	***0.28***
	All Passages	***-***	***1.14***	***0.31***

Table 4. Cytological Results from Dolphin Adipose Tissue Digestion.

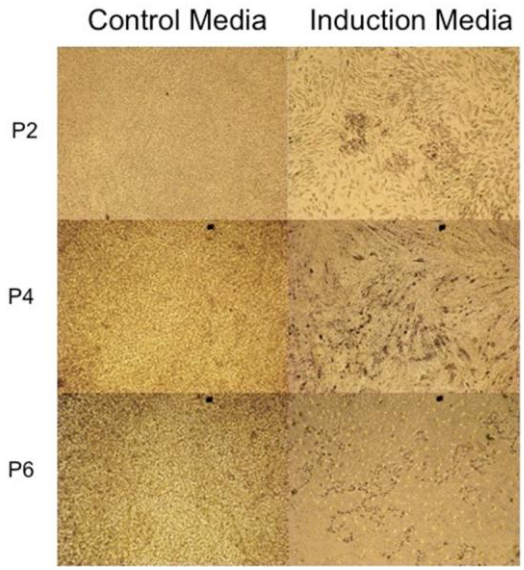
Accession Number	White Blood Cells (K/uL)	Red Blood Cells (M/uL)	Mono-nuclear Percent	Segmented Neutrophil Percent	Lymphocyte Percent	Monocyte Percent	Eosinophil Percent	Number of Platelets (K/uL)
OBA-012610-08	2.5	0.17	4	73	23	1	3	5
OBA-021710-06	3.3	0.08	8	64	28	0	0	13
OBA-022310-07	2.1	0.18	2	69	19	1	9	7
OBA-031610-11	0.6	0.23	7	62	24	3	4	2
Average	***2.13***	***0.17***	***5.3***	***67.0***	***23.5***	***1.3***	***4.0***	***6.8***
Standard Dev.	***1.13***	***0.06***	***2.8***	***5.0***	***3.7***	***1.3***	***3.7***	***4.6***

Table 5. Colony-Forming Unit-Fibroblastic (CFU-F) Assay Data.

Accession Number	*Collection Location*	*Percent CFU-f*	
		Average	*Standard Deviation*
OBA-012610-08	Nuchal Pad Liposuction	0.17%	0.07%
OBA-021710-06	Nuchal Pad Liposuction	0.19%	0.16%
OBA-022310-07	Nuchal Pad Liposuction	0.11%	0.06%
OBA-031610-11	Nuchal Pad Liposuction	0.03%	0.01%
OBA-043010-02	Necropsy - Subcutaneous Adipose	1.04%	0.24%
OBA-043010-03	Necropsy - Mandibular Adipose	0.33%	0.28%
Live Dolphin Samples Combined (n=4)		**0.13%**	**0.11%**

To better quantify the actual number of stem cells present in the dolphin adipose, processes which yielded cells of sufficient quantity were plated for CFU-f analysis. Cells are seeded in culture wells using serial dilutions and expanded for a period of 7 to 10 days to determine the percentage of cells which can replicate and form colonies. The average percent CFU-f was found to be 0.13%, about 1 adherent cell in 769 cells (Table 5). CFU-f analyses were also successfully initiated for 2 of the 3 adipose depots from the dolphin necropsy (subcutaneous and mandibular adipose) and are included in Table 5. These numbers compare with literature on human adipose CFU-f assays.

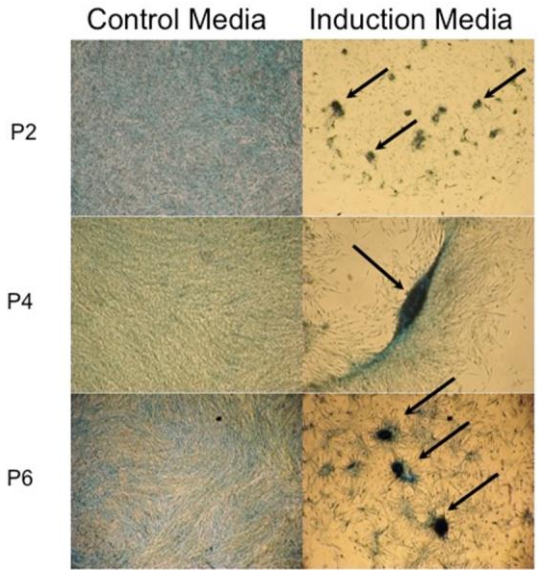
Figure 8. Adipogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.



Enough cells were present from 4 of the 6 adipose samples to perform cryopreservation of freshly isolated nucleated cells using a proprietary mixture of serum and dimethyl sulfoxide. Cryopreservation of cultured cells from all 6 dolphins has been successfully accomplished. Thus far, cryopreserved doses have been recovered from two separate dolphins; the percent recovery was 93.5% with 95.8% viable cells and 82.5% with 96.5% recovery, respectively for the two recovered doses.

Dolphin cultured cells expanded to passage 2, 4 and 6 and 4 have been successfully differentiated into multiple cell lineages. The cultured cells respond to the respective induction media and have differentiated into adipogenic (fat), chondrogenic (cartilage), osteogenic (bone) and neurogenic (nerve) cells. Photographs of these cells are shown in Figure 8 through Figure 11. In brief, cells are seeded in culture plates and expanded to the required confluence. Specific induction media is then added to the wells, with fresh media added every 3-4 days. When the required time course is complete, cells are fixed and stained for protein residues and/or cell morphology.

Figure 9. Chondrogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.



Differentiation of Cells Cultured to Passage 6

In addition to showing evidence that the cells isolated and expanded from dolphin adipose are plastic-adherent, dolphin cultured cells expanded to passage 2, 4 and 6 have been successfully differentiated into multiple cell lineages as shown in Figures 1 through 4. The cultured cells respond to the respective induction media and have differentiated into adipogenic (fat; Figure 8), chondrogenic (cartilage; Figure 9), osteogenic (bone; Figure 10) and neurogenic (nerve; Figure 11) cells as shown.

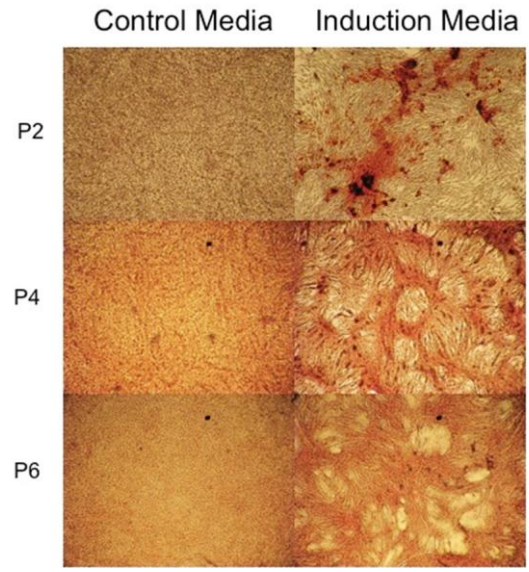
To briefly describe the procedure used in these differentiations, cells were grown to near confluence, trypsinized, seeded in culture plates and expanded to the required confluence. Induction media was then added to the wells, with fresh media added every 3-4 days. When the required time course was complete, cells were fixed and stained for protein residues and/or cell morphology specific to the respective differentiation.

The ability of these cells to differentiate at Passage 6 indicates that these cultured cells remain multipotent at later passage. Furthermore, an additional differentiation from those recommended by the ISCT has been achieved.

Characterization of the Cultured Cells through the use of Cell Surface Marker Analyses

To unequivocally determine that the cells isolated and cultured from the adipose of dolphins are in fact stem cells, fluorescent-assisted cell sorting (FACS) analysis was employed to identify key proteins present on the external surface of the cultured cells. Termed CD markers for Cluster of Differentiation, these surface markers have been previously referenced in literature to exhibit expression of these proteins on stem cells. As yet, there are no dolphin specific antibodies available and monoclonal antibodies targeted against human or canine epitopes were used. The analyses of these markers as they pertain to dolphin stem cells have focused around the more classical epitopes expressed on stem cells and those

Figure 10. Osteogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.



clearly and solidly identified in literature pertaining to stem cell related protein targets.

To briefly explain the protocol used for analysis, previously cryopreserved cultured cells from were recovered and incubated with a LIVE/DEAD dye which is used to sort viable cells from dead cells. Following a series of washes and centrifugations, cells were then incubated with isotype controls or the selected antibodies and again washed and centrifuged prior to fixation in formalin.

Figure 11. Neurogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.

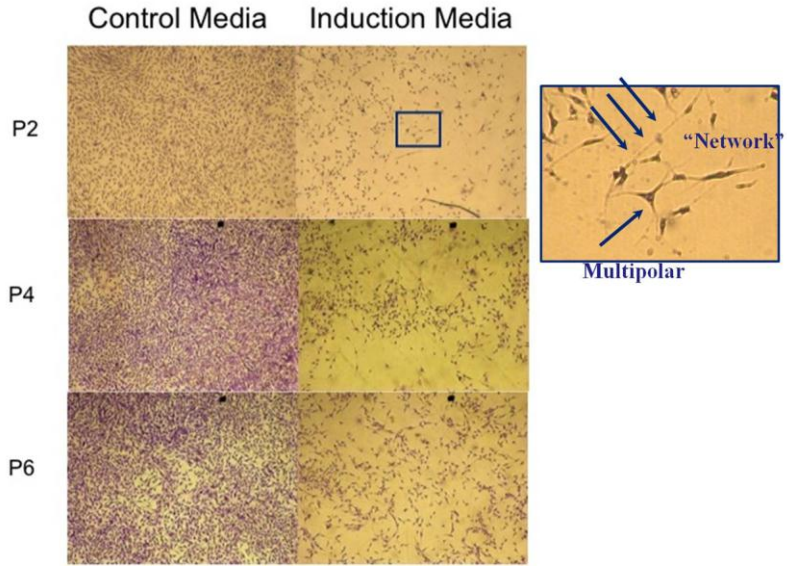
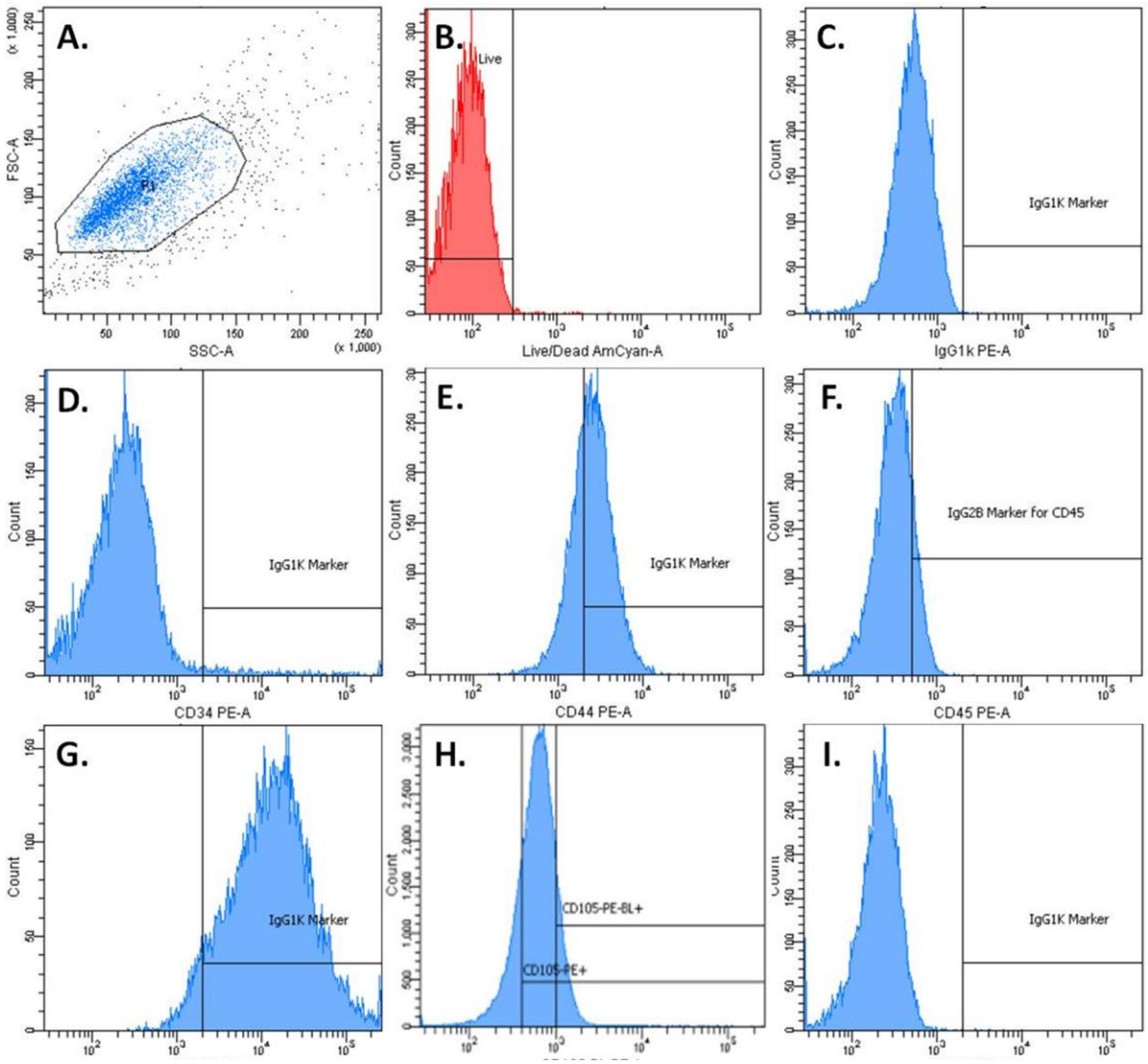


Figure 12. Representative scatter plot and histograms obtained from the FACS analysis of passage 4 cultured cells from a single dolphin. ***A. Scatter plot of forward scatter versus side scatter. B. Gating on live cells only, excluding dead cells. Histograms for: C. Isotype Control; D. CD34; E. CD44; F. CD45; G. CD90; H. CD105; I. CD271.***



The increased number of exposed amine residues on dead cells binds the LIVE/DEAD dye, making the dead cells highly fluorescent, as compared to live cells, which display a low level of fluorescent intensity. The dead cells were excluded from analysis and the live cells were analyzed for the specific antibody:fluorophore conjugate. Figure 12A-I shows a representative panel of analyses from a single dolphin at passage 4. The scatter plot in Figure 12A shows a population of relatively homogenous cells based on the forward scatter (size of the cell) versus side scatter (granularity). Throughout these analyses, the majority of the cells are viable, as shown in the gating at the top-left of Figure 12B; any cells excluded are deemed non-viable and are not analyzed for CD markers. Isotype controls were analyzed in order to set a background fluorescent threshold (Figure 12C) for CD marker expression in the dolphin cultured cells (Figure 12D-I). A similar pattern of expression was observed in 4 dolphin samples at passage 4 and in samples that were expanded to culture passage 6. The key proteins that show

expression in dolphin cultured stem cells thus far are: CD44 (hyaluran cell adhesion molecule; Figure 12E) which is a receptor for hyaluronic acid and implicated in stem cell phenotypes;

Table 6. List of antibodies that have been incubated with dolphin cultured cells.

Marker	Protein Name	Function	Antibody Raised Against	Bound to Dolphin Cultured Cells
CD29	Integrin Beta-1	Fibronectin receptor; recognition and cell adhesion	Human	-
CD34	Mucosialin	Cell-cell adhesion molecule	Canine	+/-
CD44	Hyaluran Cell Adhesion Molecule	Mediates cell adhesion and migration	Human	+
CD45	Leukocyte Adhesion Molecule	Protein tyrosine phosphatase; cell activation	Canine	+/-
CD73	Ecto-5'-nucleotidase	Catalyzes conversion of nucleotides	Human	+/-
			Human	-
CD90	Thy-1	Cell-cell interaction, adhesion and activation	Human	-
			Human	+
CD105	Endoglin	Cytoskeletal organization; cell morphology	Human	+
			Human	-
			Human	-
CD271	Low Affinity Nerve Growth Factor Receptor p75	Neurotrophin receptor; function not well defined	Human	-
			Human	-

CD90 (thymocyte-1; Figure 12G) which mediates cell-cell interaction; and CD105 (endoglin; Figure 12H) which is involved in TGF-beta signaling. The lack of expression of CD34 (mucosialin; Figure 12D) which is a hematopoietic marker has been observed to have very low expression in all cultured dolphin samples analyzed thus far; an expected result. CD44 and CD90 have shown also shown to be positive in cultured stem cells at Passage 2 and Passage 6 and prompted the use of these antibodies for immunohistochemistry.

Table 6 shows a list of the antibodies used in these studies, including the CD marker name, the function of the protein, the species the antibody was raised against and whether the antibody bound to dolphin epitopes. The utility of the CD34 and CD45 (expressed on leukocytes) antibody for use with dolphin cells could not be confirmed since samples tested with dolphin blood did not exhibit binding to the antibodies.

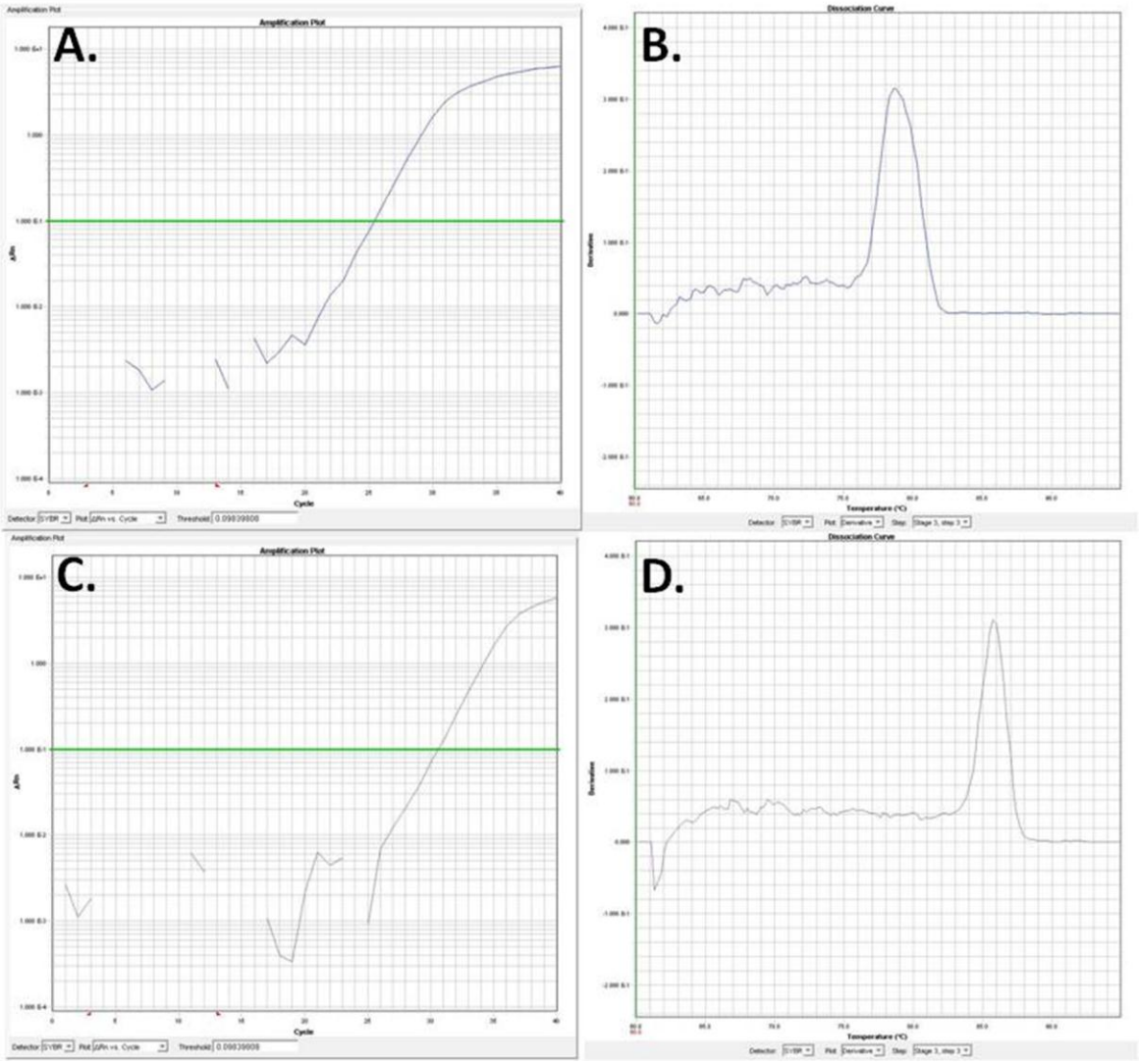
Characterization of the Cultured Cells through the use of Gene Arrays

To determine if stem cell related genes are expressed during the culture of stem cells, cells were freshly passaged and harvested for mRNA expression. In brief, the freshly harvested cells were pelleted and immediately frozen. Thawed cells were then immediately homogenized and placed on a RNA-purification column and the RNA isolated by a series of washes and centrifugation steps. Complementary DNA (cDNA) was generated and added to the real-time PCR array.

An array based on human mesenchymal stem cell genes was employed since there is little-to-no information pertaining to stem cell related genes in the dolphin. This real-time array makes use of fluorescence to monitor the live-time progression of amplification of the genes. As a gene is amplified, the fluorescence intensity increases, as seen in the amplification peaks in Figure 13A and C, which show the amplification of activated leukocyte cell adhesion molecule (ALCAM; CD166) and low-affinity nerve growth factor (LNGFR; CD271), respectively. To ensure that identified genes will have an optimal chance at positive correlation with sequences

from dolphins, the melting curves were examined to better assess the specificity. If a single

Figure 13. Representative plots of gene array data. ***Fluorescent amplification plots for A. Activated Leukocyte Cell Adhesion Molecule (ALCAM; CD166) and C. Low Affinity Nerve Growth Factor Receptor (LNGFR; CD271). Melting curve analysis for B. ALCAM and D. LNGFR.***



sequence has been amplified from the dolphin cDNA, then the melting curves will show a single peak in the melting curve analysis, as seen in Figure 13B (ALCAM) and Figure 13D (LNGFR).

Table 7 shows select genes related to stem cell proliferation. Sox2 is a transcription factor known to be implicated in maintaining pluripotency and along with Oct-4 expression is known to be a molecular indicator of embryonic stem cells. Transfection and expression of Oct-4 has also been shown to be a critical factor in the establishment of induced pluripotent stem cells. Sox9 is a transcription factor involved in chondrogenesis. While the exact function of LNGFR (CD271) is not known, antibodies raised against this marker have been used throughout the current literature to enrich adipose-derived stem cell populations. The expression of ALCAM and ICAM are both implicated in T cell activation, adherence and migration which stem cells are known to modulate.

Table 8 lists a number of genes pertaining to stem cell fate and pluripotency. Telomerase reverse transcriptase (TERT) maintains the length of the telomeres, thus maintaining pluripotency and inhibiting cellular senescence. Bone morphogenic protein 4 (BMP4) has been shown to increase stem cell survival and maintain pluripotent characteristics. Both BMP4 and BMP7 have been implicated in cell fate, such as adipo-, chondro-, osteo- and myogenic cell lineages. TGF-beta is a promiscuous protein involved in maintaining pluripotency and cell fate across multiple lineages.

Table 7. Stem cell proliferation related genes identified by gene array analysis.

Gene	*Gene Name*	*Function*
SOX2*	SRY (sex determining region Y)-box 2	Stem cell related gene.
SOX9*	SRY (sex determining region Y)-box 9	Stem cell related gene.
NGFR*	Nerve growth factor receptor; CD271	MSC surface marker.
ALCAM*	Activated leukocyte cell adhesion molecule; CD166	MSC surface marker.
ICAM1*	Intercellular adhesion molecule 1; CD54	Integrin binding receptor.
ABCB1	ATP-binding cassette, B (MDR), member 1	Transport protein.
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	MSC proliferation and differentiation.
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	MSC proliferation and differentiation.
VIM	Vimentin	Cytoskeletal marker for MSC.
FUT4	Fucosyltransferase 4	Blood group marker; present in undifferentiated MSCs.
NUDT6	Nudix (nucleoside diphosphate linked moiety X)-type motif 6	Putative proliferation gene.

Taken together, the ability for these cells to differentiate into multiple cell lineages, the expression of key CD markers and the positive expression of known stem cell related genes indicate that these cells isolated and cultured from the digestion of dolphin adipose are mesenchymal stem cells.

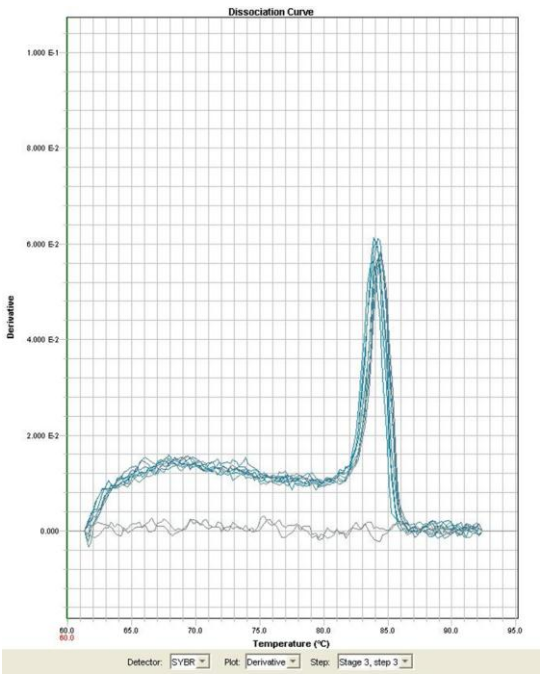
Table 8. Cell fate and pluripotency related genes identified by gene array analysis.

Gene	*Gene Name*	*Function*
TERT*	Telomerase reverse transcriptase	Maintain pluripotency.
BMP4*	Bone morphogenetic protein 4	Maintain pluripotency.
BMP7*	Bone morphogenetic protein 7	Maintain pluripotency; involved in cell fate.
TGFB1*	Transforming growth factor, beta 1	Maintain pluripotency; involved in cell fate.
EGF	Epidermal growth factor	Maintain pluripotency; involved in cell fate.
CTNNB1	beta-Catenin 1, 88kDa	Cell fate; Notch/Wnt signaling.
FGF2	basic Fibroblast growth factor 2	Maintain pluripotency; involved in cell fate.
MMP2	Matrix metallopeptidase 2 (gelatinase A, type IV collagenase)	Embryonic development, vascularization, and inflammation.
VWF	Von Willebrand factor	Maintain pluripotency; involved in cell fate.
PPARG	Peroxisome proliferator-activated receptor gamma	Maintain pluripotency; involved in cell fate.
HAT1	Histone acetyltransferase 1	Gene transcription.
JAG1	Jagged 1	Cell fate; Notch/Wnt signaling.
FZD9	Frizzled homolog 9; CD349	Cell fate; Notch/Wnt signaling.
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2; CD340	Her2; EGFR-like; cell maintenance.

PCR Verification of Select Genes

Seven gene hits from the stem cell gene array were further analyzed for verification that these genes are expressed in cultured dolphin stem cells. The expression of GAPDH (positive expression control), ALCAM, BMP7, FZD9, NGFR and SOX-9 were analyzed for positive expression in cDNA generated from the RNA of 4 separate dolphins. All of these sequences were found to be amplified in all 4 dolphins and a representative figure displaying the dissociation curve of the amplification of FZD9 in all 4 dolphins is shown (Figure 14). The amplifications of all the tested genes are to be published in a manuscript (in draft at the time of this report).

Figure 14. Verification of PCR product melting curve analysis for FZD9 from 4 separate dolphins. (showing a single, specific peak).



Autologous Wound Healing Study

Dolphins are known to acquire skin wounds through numerous modalities including propeller strikes by watercraft, shark bites and "rake" injuries (termed for the characteristic pattern of lacerations) during the establishment of natural dominance within a group. To determine if stem cells could be used in the treatment of these wounds, a wound-healing model whereby a 10 cm long x 3 mm deep incision was made on each side of the midline, craniolateral to the dorsal fin was used. The wounds were irrigated with sterile PBS for a period of approximately 10 minutes using a procedure similar to those employed by Bruce-Allen and Geraci (1985). Injections containing either freshly recovered autologous cultured stem cells in 2 ml of media with 5% autologous serum (treated) or 2 ml of media with 5% autologous serum only (control carrier solution only – no cells) were injected into a single side along the entire wound axis. Personnel were blinded to which side received stem cells or carrier only solution. After a period of 10 minutes, the animal was then returned to their pen. Throughout the study, photographs of the wounds were taken and the health of the animal was continuously monitored by veterinarians and trainers.

On Days 1, 5 and 15, an 8.0 mm circular biopsy, perpendicular to the wound axis, was taken, immediately placed in 10% formalin and labeled with the sample identity information. Prior to submission to the histopathologist, samples were transferred to pre-labeled 2 mL cryovials containing approximately 1 mL of fresh 10% neutral buffered saline. The biopsies were then embedded in paraffin, sectioned and stained with H&E (hematoxylin and eosin) and scored by a histopathologist who was also blinded to the injection status.

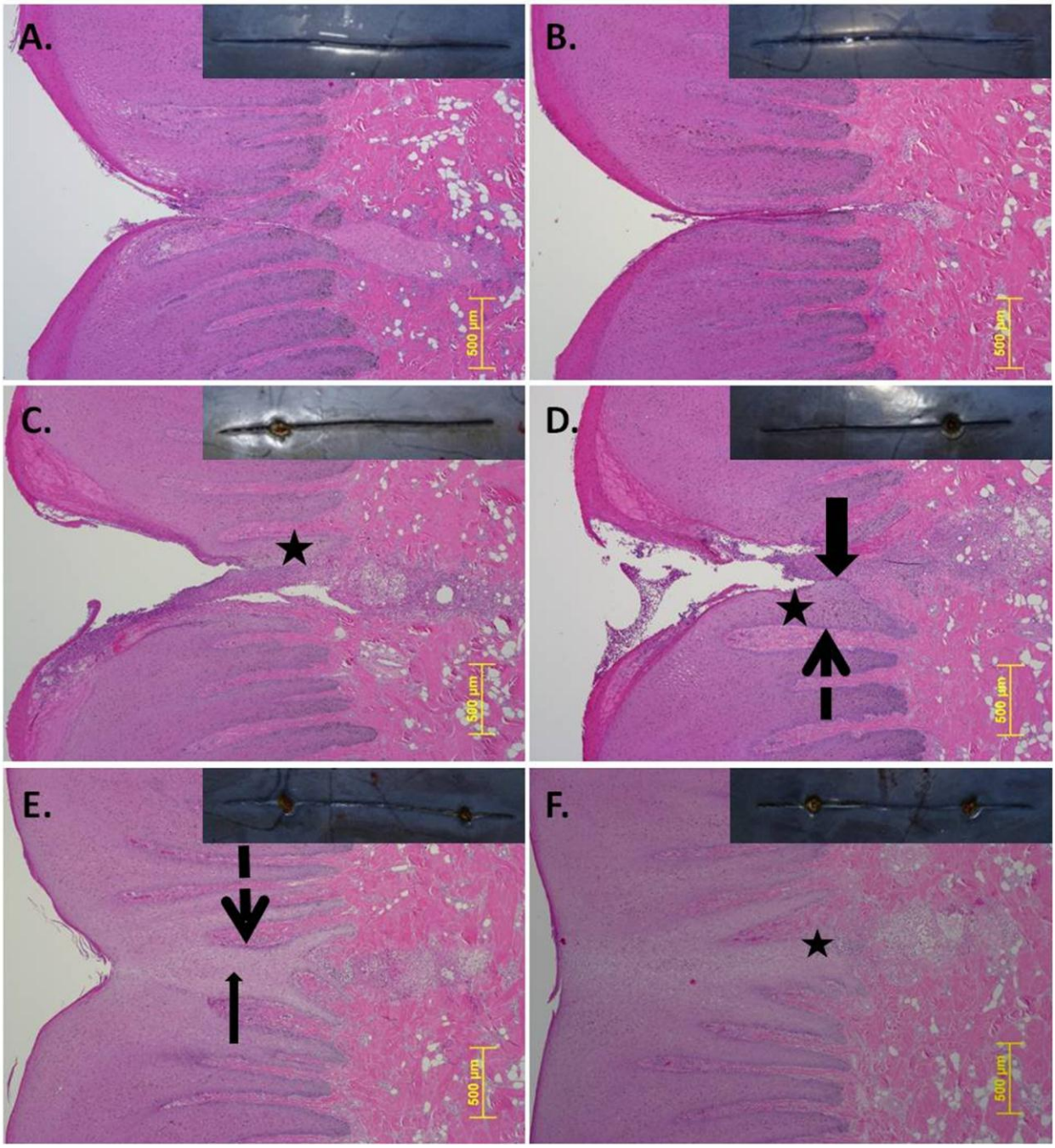
As shown in Figure 15, the left column displays the control treated side, injected only with media and 5% autologous serum (carrier solution only; A, C and E), whereas the right column shows the wound injected with stem cells and carrier solution (B, D and F). The first row of Figure 15 (A and B) shows the treatment at day 1, in which there were no qualitative or quantitative differences. By Day 5 (second row of Figure 15; C and D), a difference between the control and treated sides was noted by the histopathologist. The side treated with stem cells (Figure 15D) shows more inflammation and a greater number of mitoses suggesting more rapid cell division

and thus, healing. Additionally it was noted that there also appeared to be a more established area of re-epithelialization present in the stem cell treated side, as compared to the control treatment (carrier solution only).

By Day 15 (third row of Figure 15, E and F), the epidermal layer is completely re-established in both control and treated sides. The number of mitoses is greater and the degree of dermal inflammation is lesser in the side treated with stem cells, indicative of a more rapid rate of healing (Figure 15F) as compared to the control treated side (carrier solution only; Figure 15E). Of particular note is the qualitative observation that the side treated with stem cells has a lesser surface depression than the side treated with carrier solution alone, displaying a significant qualitative difference in healing.

These procedures have been repeated in two separate dolphins (a total of 3 separate dolphins), with the same conclusions, demonstrating and confirming that wounds treated with autologous stem cells heal at a more rapid rate. Furthermore, long-term qualitative observations (>30 days) detail that the side treated with stem cells is smoother and has less scarring than the side treated with carrier solution alone.

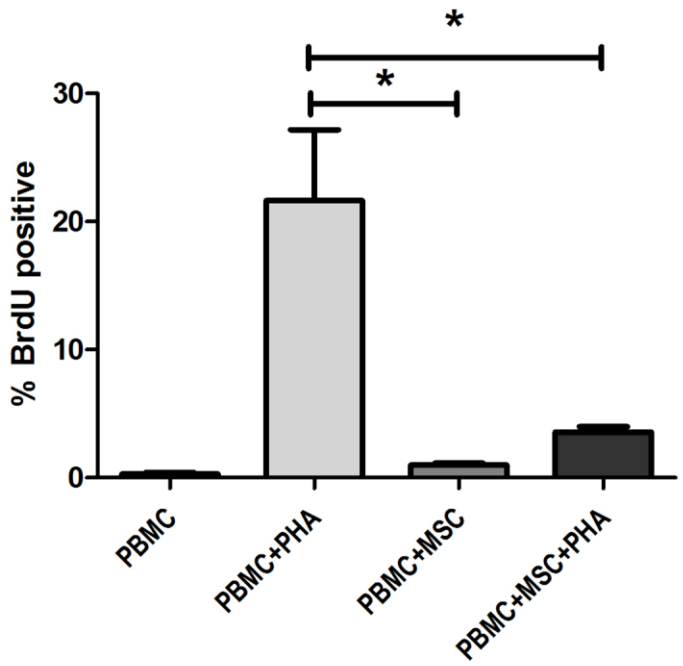
Figure 15. Histological examination of wound biopsies treated with autologous stem cells showing H&E stain along with a photograph of the wound prior to biopsy. ***Left Column is control treated (carrier solution alone – no cells); A, C, and E. Right column is the stem-cell treated; B, D, and F. Row 1 is Day 1; A and B. Row 2 is Day 5; C and D. Row 3 is Day 15; E and F. The yellow bars within each H&E stain are 500 microns across. In A and B there is no discernable difference. Stars in C and D show a subtle change between the control (C) and stem cell treated (D) sides where more epithelium has been established in the stem cell treated side, and is highlighted by the solid black arrow in D. The dashed arrow in D highlights a region proximal to the wound axis with more mitoses and rapid divisions than in C. Dashed arrow and solid arrow in E indicates a region with more inflammation and intra-dermal inflammatory cells, respectively. Black star in F indicates a region of the basal dermis with more mitoses than observed in the control injection, panel E.***



Safety of Allogeneic (different donor) Stem Cells

Vet-Stem has provided the MMP with a white paper which Vet-Stem has previously submitted to the FDA highlighting the numerous studies which demonstrate the well-established safety of allogeneic stem cells in humans and in animals. However, in order to conclusively demonstrate that allogeneic stem cells (cells from a donor dolphin) could be used to treat wounds in a non-donor dolphin, a mixed lymphocyte reaction was performed. In this assay, primary mononuclear blood cells (PBMCs; white blood cells) were isolated and incubated either alone, with phytohemagglutinin (PHA – stimulates proliferation of T-cells; a positive control for an immunogenic response), stem cells, or stem cells and PHA in combination. Proliferation of the cells is monitored by analyzing the incorporation of bromodeoxyuridine. As shown in Figure 16, when PHA is incubated with PBMCs, a large proliferative response is elicited. Allogeneic stem cells do not elicit an immunogenic response and interestingly, when stem cells and PHA are co-incubated with PBMCs, the stem cells appear to modulate the response by potentiating the proliferation of the PBMCs.

Figure 16. Results from the mixed lymphocyte reaction in dolphins.



Immunohistochemical Analysis of Wounds

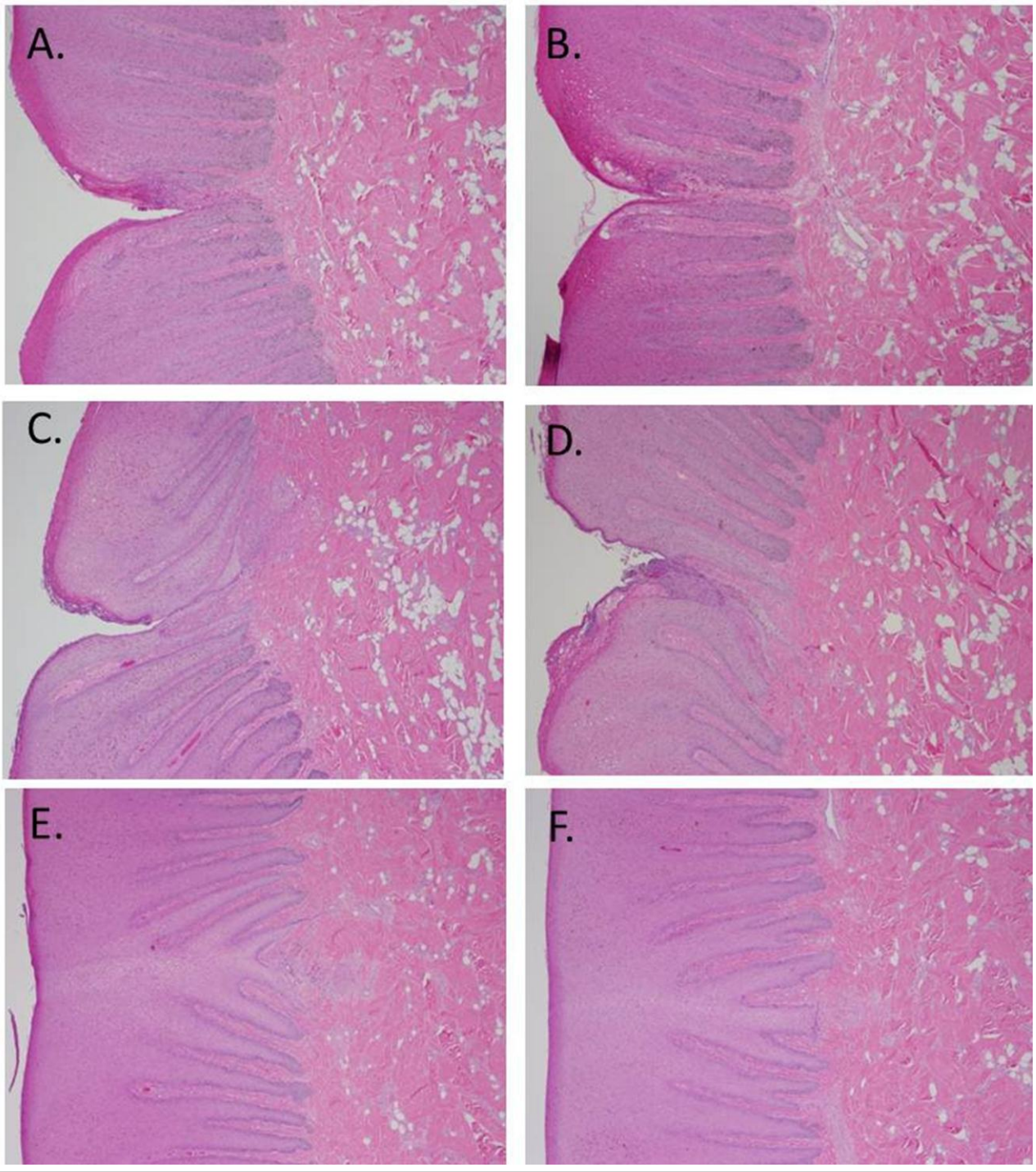
Since CD44 and CD90 antibodies have proven their utility in flow cytometry of stem cells in multiple passages,, these antibodies were also used to monitor the presence of the stem cells in paraffin-embedded tissues. In addition to standard incubation and detection techniques, antigen retrieval methods were also attempted for immunohistochemical analysis. Increasing the incubation time and increasing the antibody concentration did not yield any positive outcomes in the control tissue (thymus). There were no instances of antibody detection in any of the numerous methods and slides used.

Allogeneic Wound Healing Study

Since allogeneic stem cells did not elicit an immune response, the utility of allogeneic stem cell therapy was tested on dolphin wounds. Using the methods that are described above in Autologous Wound Healing Study, previously cryopreserved allogeneic stem cells from a single donor were resuspended in carrier solution containing media and recipient serum. While the histopathologist noted that there were minimal differences in the control (carrier solution alone) and treated (allogeneic stem cells in carrier solution) wounds, qualitative observations note that

the treated side appeared to heal quicker and the surface depressions appeared to have healed more thoroughly (Figure 17 E (control) and F (treated). In future studies, the investigators believe that a higher dose level of stem cells may yield a more prominent response, this is based upon literature citations and personal experiences with allogeneic stem cells in the canine species.

Figure 17. Histological examination of wound biopsies treated with allogeneic stem cells showing H&E stain. ***Left Column is control treated (carrier solution alone – no cells); A, C, and E. Right column is the stem-cell treated; B, D, and F. Row 1 is Day 1; A and B. Row 2 is Day 5; C and D. Row 3 is Day 15; E and F.***



References:

Dominici M; Le Blanc K; Mueller I; Slaper-Cortenbach I; Marini FC; Krause DS; Deans RJ; Keating A; Prockop DJ; Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular therapy position statement. *Cytotherapy*, (2006) Vol. 8, No. 4, pp. 315-7.

Bruce-Allen LJ and Geraci JR. Wound healing in the bottlenose dolphin (Tursiops truncatus). *Canadian Journal of Fisheries and Aquatic Sciences*, (1985) Vol. 42, pp. 216-228.

Catania JM and Harman RJ. Assessment of regenerative capacity in the dolphin. Defense Technical Information Center. Accession number: ADA530626.

e. Conclusions

A suitable technique and has been developed whereby stem cells can be harvested in a non-invasive manner from an adipose depot in the dolphin. Cells isolated from the digestion of adipose are plastic-adherent, differentiate into multiple cell lineages and express key cell surface markers; satisfying the criteria set forth by the International Society for Cell Therapy. Furthermore, these culture-expanded stem cells hasten the healing of wounds when used autologously. When incubated with white blood cells from a separate donor, the cultured expanded stem cells do not elicit an immunogenic response. Allogeneic stem cells were used to treat wounds and were qualitatively observed to hasten the wound healing process. Work presented in this report describes the first conclusive evidence of adipose derived stem cells in the Atlantic bottlenose dolphin.

f. Future Directions

Future directions of this research should focus on key areas to try to determine the cellular fate of the stem cells after they are injected into the wound axis. This would require loading the stem cells with iron to easily visualize how the cells are able to migrate throughout the wound. Since the allogeneic cells have been shown to be non-immunogenic, these cells could be used clinically to treat a number of disease states, including diseases of the liver and kidney.

g. Major Problems/Issues

There are limited numbers of antibodies and genes that have proven useful in dolphin cells. While the studies presented herein have helped to further the analyses and confirmation of stem cells in this marine mammal, antibodies and gene primers may not perform as expected in embedded tissue.

h. Technology Transfer

This ONR-funded project has provided technology to Vet-Stem in some of the following internally-funded programs:

Clinical trials in allergic skin disease.

Further characterization of stem cell markers useful for equine and canine applications.

Vet-Stem is proceeding with the development of an advanced adipose collection device, partially as a result of the liposuction procedural difficulties encountered with dolphins. This device is currently being tested in equine and canine samples.

Additional commercial cases from animals with skin disease or diseases with related mechanisms of progression have benefited. These include renal, cardiac, inflammatory bowel syndrome and liver disease.

Vet-Stem has established pilot cooperative research and development agreements with Dr. John Morley of St. Louis University. This cooperative research agreement was a result of the 2010 Office of Naval Research Biosciences Program Review. An initial study of rodent adipose has been completed; the next phase will involve the use of cell therapy treatment for rodents with Alzheimer's disease.

Through interactions at the ONR Biosciences review program, there have been multiple fruitful interactions for studies that are in the process of being realized as potential treatments, such as epilepsy.

There are no plans for Technology Transfer of ONR-funded research and development in the upcoming year.

i. Foreign Collaboration and Supported Foreign Nationals

None; not applicable.

j. Productivity

Journal articles/book chapters/technical reports:

a. "Adipose-derived stem cell collection and characterization in Bottlenose dolphins (Tursiops truncatus)." SP Johnson, JM Catania, RJ Harman and ED Jensen. *Stem Cells and Development*. Accepted and available online as of June 4 2012.
b. "Assessment of Regenerative Capacity in the Dolphin." Annual technical report. October 10, 2011. Defense Technical Information Center accession number ADA550347.
c. "Assessment of Regenerative Capacity in the Dolphin." Annual technical report. October 10, 2010. Defense Technical Information Center accession number ADA530626.
d. "Characterization of dolphin adipose derived stem cells" JM Catania, SP Johnson, ED Jensen and RJ Harman. Currently in final draft. Expected publication in mid to late 2012.
e. "Adipose-derived stem cells for autologous and allogeneic wound healing from bottlenose dolphins." SP Johnson, JM Catania, MJ Kinsel, DD Carrade, DL Borjesson, RJ Harman, ED Jensen. Expected publication late 2012 to early 2013.

Workshops and Conferences:

a. International Association for Aquatic Animal Medicine; Cellular characterization of adipose derived stem cells isolated from bottlenose dolphins (Tursiops truncatus); Contributing Speaker: Robert J. Harman; Atlanta, GA; May 16, 2012.
b. International Association for Aquatic Animal Medicine; Effects of autologous adipose-derived stem cells on wound healing in the bottlenose dolphins (Tursiops truncatus); Contributing Speaker: Shawn P. Johnson; Atlanta, GA; May 16, 2012.
c. International Association for Aquatic Animal Medicine; Collection of Adipose-derived Stem Cells in Bottlenose Dolphins; Contributing Speaker: Shawn P. Johnson; Las Vegas, NV; May 12, 2011.
d. ONR Marine Mammal Health/Pain/Antibiotics/Pain Program Review; Assessment of Regenerative Capacity in the Atlantic Bottlenose Dolphin; Invited Speaker: Jeffrey M. Catania; Arlington, VA; June 27, 2011.
e. ONR Marine Mammal Health/Pain/Antibiotics/Pain Program Review; Assessment of Regenerative Capacity in the Atlantic Bottlenose Dolphin; Invited Speaker: Robert J. Harman; Arlington, VA; June 9, 2010.
f. Navy Marine Mammal Program Summit 2010; Regenerative Cells: Use of Adult Stem Cells in Marine Mammals; Invited Speaker: Robert J. Harman; San Diego, CA; March 23, 2010.

Patents/Inventions:

a. Not applicable.

Awards/Honors:

a. Not applicable.